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TECHNICAL MANUSCRIPT 499

A PLAQUE ASSAY FOR RICKETTSIA RICKETTSII

Edmund H. Weinberg
Jack R. Stakebake
Peter J. Gerone

APRIL 1969

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

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Virus & Rickettsia Division
BIOLOGICAL SCIENCES LABORATORIES

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April 1969

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

ABSTRACT

A plaque technique for the assay of Rickettsia rickettsii is described. The method employs primary chick or African green monkey kidney (VERO) monolayer cell cultures with either an agarose or a special Noble agar overlay. Plaques were counted after 6 days; resultant titers correlated well with LD_{50} end points obtained by a standard assay in embryonated eggs. The plaque-forming organisms were identified by direct observation of rickettsia-like bodies in the monolayer lesions, inhibition of plaques by antibiotics, sensitivity of plaques to specific immune serum, and failure to cultivate other microorganisms from the infected cells. Versatility of the test was demonstrated by assaying samples of rickettsiae from several different sources commonly used in our laboratory. These included infected yolk sacs, various cell cultures, and infected guinea pig tissue. Sufficient numbers of viable rickettsiae were present in the cells of a single lesion to permit direct recovery.

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I. INTRODUCTION*

For many years, investigators working with rickettsiae have recognized a need for improved methods of assay. The usual assay employing embryonated eggs has many attendant difficulties, including high variability due to factors that are often difficult to control. The egg inoculation assay requires 10 or 11 days and does not have the advantages inherent in an enumeration procedure. In 1966, Kordova** described a plaque technique that she applied to several species of rickettsiae but she did not include Rickettsia rickettsii. She reported several serious limitations of the technique including failure to obtain plaques in 16 of 22 attempts. The test required 14 to 17 days for completion, and the end points were 100-fold less sensitive than those obtained by the egg inoculation procedure. The present report describes a plaque technique for R. rickettsii that is as sensitive as the yolk sac assay, takes much less time, and is highly reproducible.

II. MATERIALS AND METHODS

A. RICKETTSIA

The organism under study was the Bitter Root strain of R. rickettsii in 68th passage level in chick embryo (CE) yolk sacs.

The working seeds were prepared by homogenizing the infected yolk sacs in a blender. The homogenate was centrifuged at 6,000 rpm in a refrigerated centrifuge. The pellet was homogenized in a Ten Broeck grinder, and following centrifugation at 1,500 rpm the supernatant fluid was removed and the pellet was discarded. The supernatant fluid was centrifuged at 6,000 rpm and the pellet was resuspended in sucrose phosphate buffer,*** dispensed in glass ampoules, and stored in a mechanical deep freeze until used. These seeds had titers of approximately $1 \times 10^{7.5}$ to $1 \times 10^{8.0}$ yolk sac 50% lethal doses per ml ($YSLD_{50}$/ml).

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* This report should not be used as a literature citation in material to be published in the open literature. Readers interested in referencing the information contained herein should contact the senior author to ascertain when and where it may appear in citable form.

** Kordova, N. 1966. Plaque assay of rickettsiae. Acta Virol. 10:278.

*** Weiss, E.; Rees, H.B.; Hayes, J.R. 1967. Metabolic activity of purified suspensions of Rickettsia rickettsi. Nature 213:1020-1022.

Additional sources of the organisms were obtained by infecting confluent monolayers of 24-hour primary chick fibroblasts, VERO cells (an African green monkey kidney cell line), or HK cells (a hamster kidney cell line) with the working seed. Propagation of rickettsiae in these systems was initiated by first removing the cell attachment fluid, applying 0.1 ml of working seed to the cells for 15 minutes at room temperature, and then, without removing the excess inoculum, adding 5 ml of medium 199 (M199) supplemented with 5% calf serum (CaS). The monolayers were then incubated in a closed system at 33.5 C until cytopathic changes were seen in 95% of cells observed under low-power magnification. The yield was harvested by suspending the cells in the supernatant fluid with the aid of glass beads and then subjecting the suspension to rapid passage through a 25-gauge needle three times. This procedure ruptured many cells and dispersed clumped cells and rickettsiae. Disruption of the cells by sonic cavitation offered no additional advantages. Rickettsiae from infected guinea pig testicles and tunica albuginea were released by first mincing the tissues with scissors and then homogenizing them in M199 plus 5% CaS with a Ten Broeck grinder. Before the inoculum was used, the lipoidal material that collected on the surface was aspirated and discarded.

B. PLAQUE PROCEDURE

The diluent for all inocula was either cold brain heart infusion broth (BHIB) or M199 containing 5% CaS. The cell attachment fluid was removed from the 24-hour confluent chick primary monolayers, and 0.1 ml of the inoculum was applied to the cells for 15 minutes at room temperature with the bottles* tightly sealed. During this time care was taken to assure that the inoculum contacted the entire sheet. Tilting the bottle at several angles immediately after application of the inoculum dispersed the organisms. An agar overlay consisting of M199, 5% CaS, and either 0.5% agarose** (firm agar) or 0.15% special agar-Noble*** (semisolid agar) was then applied.

When 100 ml of overlay were to be prepared, the proper amount of agar was melted in 10 ml of water at 10 lb. pressure for 10 minutes. Ninety milliliters of medium at 56 C were added to the liquid agar, which was then kept at 56 C and cooled to 40 C just before use. After the overlay had formed a gel over the cells, the closed bottles, not inverted, were incubated at 32 C for 6 days.

The plaques that formed were of sufficient contrast to be counted by using indirect illumination of the cell sheet while holding the bottle above a dark background. Although no stain was needed, improved contrast was achieved by applying 1 ml of a 0.1% neutral red solution on the firm agar and permitting diffusion to the cells for 1 hour at 37 C. When semisolid agar was used, the overlay was first removed by inclining the

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* Plastic flask, 30 ml, Falcon Plastics, Los Angeles, California.
** Agarose-special grade, Mann Research Laboratories, New York, N.Y.
*** Difco Laboratories, Detroit, Michigan.

bottle and sharply tapping the side. This liquefied the agar, which was then decanted. One milliliter of neutral red solution was applied to the cells for 5 minutes at 37 C, the excess was decanted, and after 30 minutes' further incubation at 37 C the plaques could be observed without special illumination.

Rickettsiae were isolated from a single plaque by either aspirating the cells with a curved Pasteur pipette or by removing them with a bacteriological loop. The cells were suspended in 0.2 ml of M199 with 5% CaS and applied to another monolayer of chick fibroblasts.

III. RESULTS

A. PLAQUE MORPHOLOGY

Plaques formed by R. rickettsii in CE monolayers after the 6th day of infection are shown in Figure 1. The plaques were about 2 mm in diameter with relatively little size variation. Incubation beyond 6 days resulted in enlargement of the plaques to about 3 mm. Nonspecific deterioration of the entire monolayer occurred after the 8th day of incubation.

Plaques that developed under the semisolid (0.15%) special Nobie agar (Fig. 1) frequently had central accumulations of cell debris that were visible macroscopically. Plaques in monolayers under firm (0.5%) agarose were opalescent and of poorer contrast and showed no cellular accumulations in their centers. Recent experiments suggest that this observed difference in morphology is related to the kind of agar used rather than the difference in agar concentration. When CE monolayers were used, the plaques were sufficiently distinct to be counted without staining. Neutral red staining was used to improve the contrast for photographic purposes.

B. PLAQUE COUNTS

The plaque counts from three 10-fold dilutions are given in Table 1. As expected, the inoculum dilution and the plaque count were directly correlated. In a second experiment, twofold dilutions of a rickettsial suspension were assayed by the plaque method. The results in Figure 2 show the direct proportionality between the plaque count and the relative concentration in the inoculum.

Plaque counts of several rickettsial suspensions were compared with the standard egg LD_{50} end points. Rickettsiae grown on three different substrates were used in these comparative tests. The plaque-forming units (pfu) and $YSLD_{50}$ end points were similar (Table 2) and the differences were not significant at the 5% level.

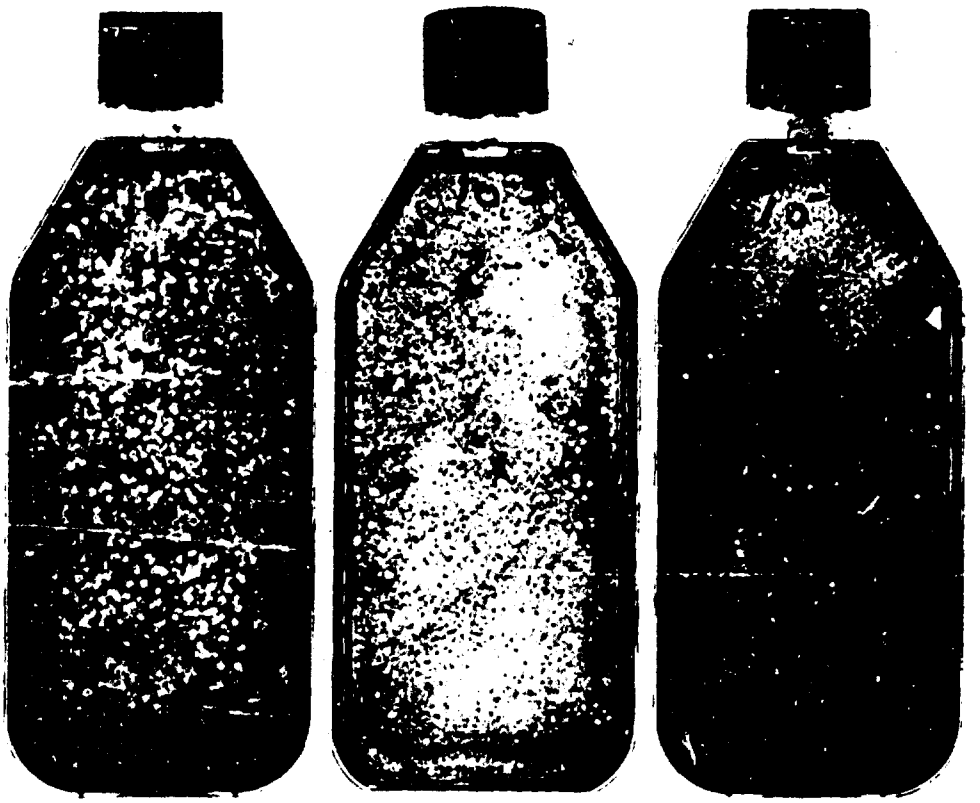

FIGURE 1. Plaques in Chick Primary Cells Due to Rickettsia rickettsii. Three tenfold dilutions (10^{-4}, 10^{-5}, 10^{-6}) of a suspension that contained $10^{8.4}$ $\log_{10}$ $YSLD_{50}$/ml are shown.

TABLE 1. PLAQUE COUNTS OF RICKETTSIA RICKETTSII ON PRIMARY CHICK EMBRYO CELL CULTURES

Dilution of Inoculum[a]	Plaque Counts/ml	Suspension Titer per ml x 10^7
10^{-7}	2, 4, 1	2.3
10^{-6}	21, 20, 24	2.17
10^{-5}	164	1.64

a. Rickettsial suspension grown in CE cell cultures containing 1 x $10^{7.5}$ $YSLD_{50}$/ml.

C. EFFECTS OF VARIABLES AND PROCEDURAL MODIFICATIONS

M199 containing 5% calf serum and BHIB were compared as diluents for the plaque assay on CE cells. The data in Table 3 show both diluents to be equally suitable with two different rickettsial seeds. The rickettsiae were stable for several hours at 4 C in both diluents. Rickettsiae stored at -60 C in skimmed milk could be readily assayed by the plaque method if sufficiently diluted in BHIB so that the opacity of the milk did not obscure the plaques.

Agar overlays of 5 ml and 10 ml were compared using two seeds of R. rickettsii. The results shown in Table 4 suggested that the 10-ml overlay was slightly better, but the difference was not statistically significant.

In another experiment, plaques were produced under various concentrations of methylcellulose, with 0.5% in M199 being optimal. The use of methylcellulose offered no advantage over the agar overlays and because of its difficulty in handling it was not used thereafter.

The incorporation of either 100 or 200 μg of DEAE dextran per ml of 0.5% agarose or 0.5% special Noble agar overlay had no effect on either plaque size or number. The monolayers under the dextran overlays deteriorated after 6 days, compared with 8 days for the controls without the dextran. The higher concentration of dextran affected the monolayer so severely that the plaques were difficult to differentiate.

The length of time the inoculum was allowed to contact the CE monolayers was varied from 15 to 60 minutes. The results (Table 5) indicated that adsorption for 15 minutes was adequate because the plaque counts were approximately equal for all three time periods tested.

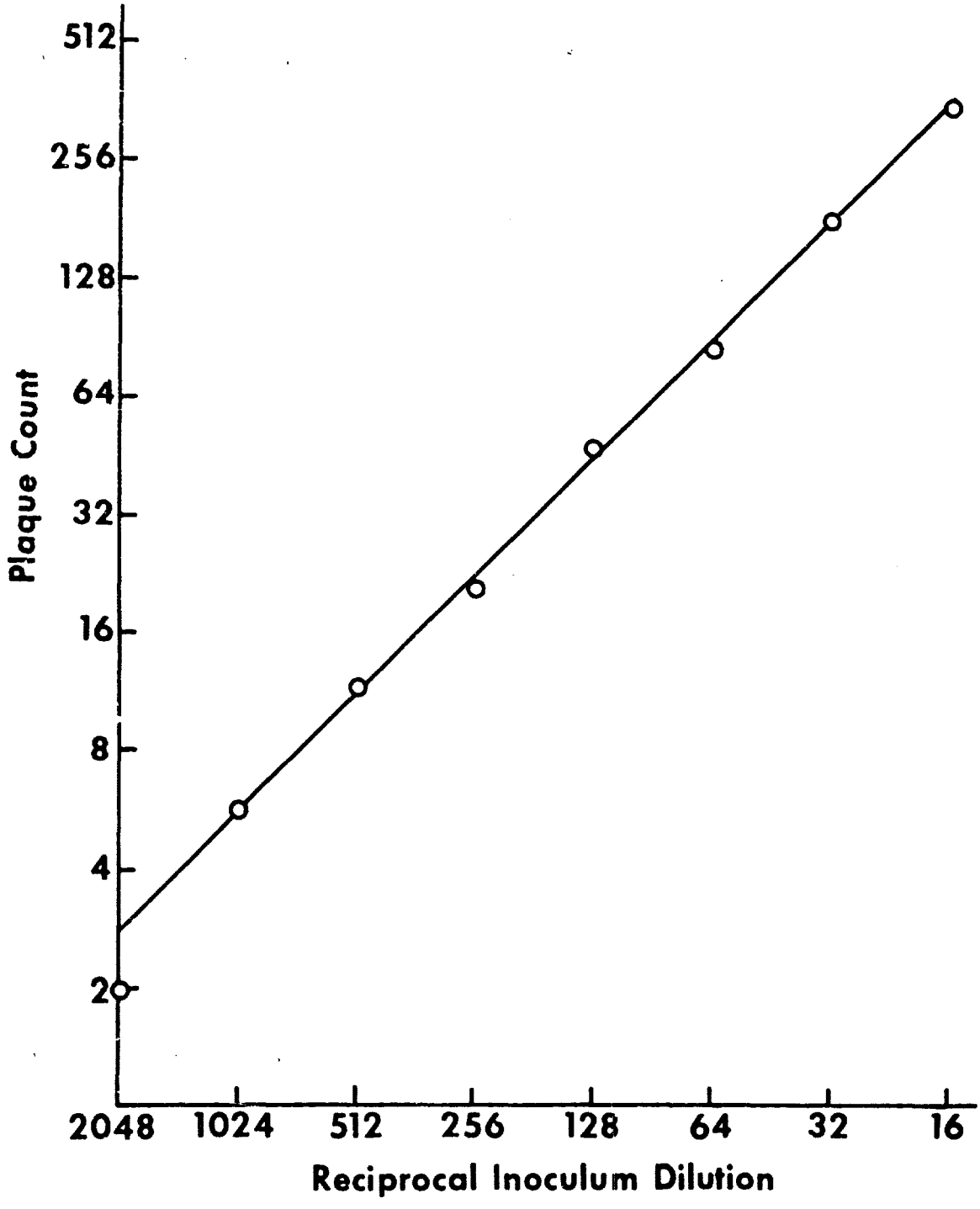

FIGURE 2. Relationship Between Plaque Count and Inoculum Dilution.

TABLE 2. COMPARISON OF SENSITIVITY OF PLAQUE AND EGG ASSAY TECHNIQUES

Source of Rickettsiae	Titer (log_{10}) Plaque, pfu/ml	Egg, $YSLD_{50}$/ml
L cell culture	4.4	4.1
	4.3	4.9
	5.1	5.4
	5.6	5.6
	5.4	5.4
	6.0	6.2
VERO cell culture	4.7	5.0
	3.7	3.8
	4.8	4.9
	6.0	6.0
	5.9	6.0
	5.5	5.5
	5.3	5.5
	4.6	5.5
Yolk sac	7.8	7.6
Mean difference	0.18	
Standard deviation	0.28	

TABLE 3. EFFECT OF DILUENT ON SENSITIVITY OF THE PLAQUE TEST

Dilution of Inoculum[a]	Seed I Counts of Plaques M199-CaS[b]	Seed I Counts of Plaques BHIB[c]	Seed II Counts of Plaques M199-CaS	Seed II Counts of Plaques BHIB
10^{-5}	31;25	47;18	35;56;44	31;25;60
10^{-6}	5;5	5;3	5;1;3	1;5
10^{-7}	1;0	0;0	1;0;0	0;0;0

a. Suspension of R. rickettsii obtained from infected CE cell monolayers.
b. Medium 199 with 5% calf serum.
c. Brain heart infusion broth.

TABLE 4. EFFECT OF VOLUME OF OVERLAY ON PLAQUE TEST SENSITIVITY[a]

Dilution of Inoculum[b]	Sample A		Sample B	
	10 ml	5 ml	10 ml	5 ml
10^{-2}	TNTC[c]	TNTC	TNTC	TNTC
10^{-3}	56;68	47;51	40;37	18;32
10^{-4}	13;7	3;5	5;8	3;8
10^{-5}	1;0	1;0	1;1	1;0

a. Medium 199, 5% calf serum mixed with agarose to make a 0.5% agar overlay.
b. Suspension of R. rickettsii grown in hamster kidney cells.
c. Too numerous to count.

TABLE 5. EFFECT OF ADSORPTION TIME ON PLAQUE ASSAY SENSITIVITY

Dilution of Inoculum[a]	Adsorption Time[b]					
	15 Minutes		30 Minutes		60 Minutes	
	Counts	Mean	Count	Mean	Counts	Mean
10^{-5}	55;50;45 60;35;53	50	44;35;46 45;57;56	47	53;52;52 54;39;40	48
10^{-6}	3;1;6 4;8	4.4	10;5;3 5;2	5.0	4;1;5 0;1	2.2
10^{-7}	0;1;0 1;0		2;0 0;0;0		0;1 0;0;0	

a. Partially purified rickettsial suspension. Yolk sac source containing 7.4 $\log_{10}$ $YSLD_{50}$/ml.
b. Interval between application of 0.1 ml inoculum and addition of the overlay.

Attempts were made to produce plaques with R. rickettsii in monolayers of VERO, hamster kidney, L, and cat kidney cell lines. Only the VERO cells showed plaques. The plaques were equal in size and number to those observed on CE monolayers but were of poorer contrast. It was necessary to apply a neutral red stain to facilitate counting the plaques. The poor contrast was probably explained by the observation under the microscope that there were many normal-appearing cells within the plaque boundary; the affected cells appeared rounded and granular.

D. SPECIFICITY OF THE PLAQUES

Several observations provide substantial evidence that the plaques formed in the primary CE monolayers were a direct result of R. rickettsii. When cells obtained from a plaque were stained with carbolfuchsin and counterstained with malachite green, numerous intracytoplasmic rods consistent in morphology with R. rickettsii were seen. No such rods were seen within the cytoplasm of cells outside the plaque perimeter.

When 0.5 ml of specific immune serum (complement fixation titer, 1:512) of rhesus monkey origin was added to the overlay medium of each bottle, the plaque size was reduced to an average of 0.5 to 0.75 mm, and the plaque borders were no longer uniform and sharply demarcated. The plaque numbers, however, were not reduced.

Addition of a mixture of antibiotics (100 units of crystalline penicillin and 50 µg of streptomycin per ml) to the overlay reduced the plaque size to "pinpoints" in the three lowest inoculum dilutions tested and completely inhibited plaque formation at the eight higher dilutions (Table 6). Chlortetracycline (0.5 µg/ml of agar) totally inhibited the formation of plaques at all 11 inoculum dilutions tested.

Cell culture samples obtained from dense plaque areas were inoculated into BHIB, thioglycolate broth, and a mycoplasma culture broth. The samples were also streaked on BHIB-agar and mycoplasma plates. All cultures were incubated at two temperatures (32 C and 37 C) and in aerobic conditions, anaerobic conditions, or under 5% CO_2 concentrations. All cultures failed to reveal the presence of other organisms. One milliliter of the inoculum used to form plaques was injected into guinea pigs intraperitoneally in a concentration of $1 \times 10^{3.4}$ $YSLD_{50}$/ml diluted in isotonic saline. Additional portions of the sample were titrated in embryonated eggs and by the plaque method on CE cells. The febrile and scrotal responses in guinea pigs and the mortality pattern in eggs were typical of reactions normally produced by R. rickettsii.

TABLE 6. INFLUENCE OF PENICILLIN AND STREPTOMYCIN ON PLAQUE COUNTS AND MORPHOLOGY

Dilutions of a Suspension Containing $10^{3.4}$ $YSLD_{50}$/ml	Counts with Antibiotics [a]	Counts without Antibiotics
1:2	TNTC,[b] PP[c]	TNTC
1:4	TNTC, PP	TNTC
1:8	5, PP	TNTC
1:16	0	367
1:32	0	190
1:64	0	98;87
1:128	0	60;45
1:256	0	25;20
1:512	0	14;11
1:1024	0	7;5
1:2048	0	3;1

a. 100 units potassium penicillin/ml and 50 µg streptomycin sulfate per milliliter added to the overlay.
b. Too numerous to count.
c. Pinpoint-size plaques.

IV. DISCUSSION

The usefulness of plaque assay techniques based on the ability of organisms to produce focal lesions in monolayer or suspensions of cells in agar has been well documented with many viruses. Plaque procedures are considerably more versatile as research tools than quantal assays based on the presence or absence of signs of infection or death in a host. Accordingly, the development of a plaque assay for R. rickettsii would facilitate research with this organism.

Examination of several variables and procedural modifications showed little or no effect on the numbers of plaques formed. Both M199 containing 5% calf serum and BHIB gave similar results. Ten-milliliter agar overlays produced counts that were slightly higher than those under 5-ml overlays, but the difference was not statistically significant. Plaques formed equally well under agar, agarose, or methylcellulose. The incorporation of 100 to 200 μg of DEAE dextran per ml of agar or agarose did not alter the size or number of developing plaques. Results from the experiments in which the adsorption time varied indicated that rickettsiae attached to the cells within 15 minutes or that adsorption could take place after the overlay was applied.

Several experimental approaches were used to establish the specific relationship between the observed plaques and R. rickettsii. The drastic reduction of plaques by the incorporation of antibiotics into the overlay virtually eliminates the possibility that the lesions were caused by some unknown, contaminating virus. The failure to recover organisms from the inoculum by aerobic as well as anaerobic culture techniques tends to eliminate bacteria from consideration.

Because mycoplasma are frequent contaminants of embryonated eggs and cell cultures, are sensitive to many antibiotics, are sometimes difficult to isolate, and have been known to cause plaques in cell cultures,* organisms of this group were more difficult to eliminate as agents responsible for the observed plaques. The experimental evidence that suggests that the plaques were not caused by mycoplasma includes the following: (i) the plaques were produced by an organism highly sensitive to a combination of penicillin and streptomycin. Most known mycoplasma are not sensitive to either antibiotic, and R. rickettsii is known to be sensitive to streptomycin. (ii) When samples from growth studies in cell culture were assayed by both the plaque test and by embryonated egg inoculation, the pfu and $YSLD_{50}$ end points were similar. If mycoplasma were responsible for the plaques, one would have to postulate either an uncommon virulence of

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* Zgorniak-Nowosielska, I.; Sedwick, W.D.; Hummeler, K.; Koprowski, H. 1967. New assay procedure for separation of mycoplasma from virus pools and tissue culture systems. J. Virol. 1:1227-1237.

mycoplasma for eggs that mimics the pathogenicity of R. rickettsii or that growth of mycoplasma and rickettsiae in the cell cultures proceed at the same rate and to equivalent yields. (iii) When an inoculum that produced plaques was injected into guinea pigs, the animals showed typical signs of R. rickettsii infection leading to death. Further, a suspension of testicular tissue including the tunica albuginea taken at autopsy produced plaques and was lethal for eggs to the same titer. If the plaques had been produced by mycoplasma, one would also have to argue that the mycoplasma infected the guinea pigs and was found in the testicular tissue in the same concentration as the rickettsiae.

Additional evidence that suggested that the plaques were formed specifically by R. rickettsii was based on the presence of rickettsia-like bodies in the cytoplasm of stained cells within the perimeter of the plaque. Moreover, immune serum taken from a monkey infected with R. rickettsii caused a reduction in plaque sizes when incorporated into the overlay medium.

The results strongly suggest that the plaques observed in these experiments were formed by R. rickettsii. As an assay procedure, the plaque test is more economical, requires less time, has less variability, and is more versatile than the conventional assay procedure involving embryonated eggs. Recent studies in this laboratory have already adapted this technique to testing the organism's sensitivity to antibiotic discs. Recovery of the organism from single plaques makes it possible to perform genetic studies with R. rickettsii that were not previously possible. Experiments are under way to develop a neutralization test based on plaque inhibition.

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13 ABSTRACT

A plaque technique for the assay of Rickettsia rickettsii is described. The method employs primary chick or African green monkey kidney (VERO) monolayer cell cultures with either an agarose or a special Noble agar overlay. Plaques were counted after 6 days; resultant titers correlated well with LD_{50} end points obtained by a standard assay in embryonated eggs. The plaque-forming organisms were identified by direct observation of rickettsia-like bodies in the monolayer lesions, inhibition of plaques by antibiotics, sensitivity of plaques to specific immune serum, and failure to cultivate other microorganisms from the infected cells. Versatility of the test was demonstrated by assaying samples of rickettsiae from several different sources commonly used in our laboratory. These included infected yolk sacs, various cell cultures, and infected guinea pig tissue. Sufficient numbers of viable rickettsiae were present in the cells of a single lesion to permit direct recovery.

14. Key Words

*Rickettsia rickettsii
*Assaying
*Identification

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